Estrogens Inhibit Amyloid-β-Mediated Paired Helical Filament-Like Conformation of Tau Through Antioxidant Activity and miRNA 218 Regulation in hTau Mice

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(Article begins on next page)

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Abstract.
Background: The risk of developing Alzheimer's disease as well as its progression and severity are kno
men and women, and cognitive decline is greater in women than in men at the same stage of disease an
at least in part on estradiol levels.
Objective: In our work we found that biological sex influences the effect of amyloid-β_{42} (Aβ_{42}) mono
tau conformational change.
Methods: In this study we used transgenic mice expressing the wild-type human tau (hTau) whic
intraventricular (ICV) injections of Aβ peptides in nanomolar concentration.
Results: We found that Aβ_{42} produces pathological conformational changes and hyperphosphorylat
male or ovariectomized female mice but not in control females. The treatment of ovariectomized fe
replacement protects against the pathological conformation of tau and seems to be mediated by antiox
as the ability to modulate the expression of miRNA 218 linked to tau phosphorylation.
Conclusion: Our study indicates that factors as age, reproductive stage, hormone levels, and the inte
factors should be considered in women, in order to identify the best appropriate therapeutic approach in pr
impairment.

Keywords: Alzheimer's disease, antioxidants, estradiol, miRNA, tau protein

INTRODUCTION

Hallmarks of Alzheimer's disease (AD) are the accumulation of amyloid-β (Aβ) peptides in amyloid plaques, and the aggregation
form neurofibrillary tangles.

Aβ derives from the amyloid
sor (AβPP) through β site APP
(BACE) 1 and γ-secretase proc
ates multiple C-termini, most en
and 42. Aβ_{42} aggregates more
bly than Aβ_{40} through sequentia

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which were subjected to intraventricular (ICV) injections of Aβ peptides in nanomolar concentration. We discovered that $A\beta_{42}$ monomers, but not oligomers: 1) produce paired helical filament-like conformation of tau protein, and 2) induce two phosphorylated epitopes which are not present in normal tau (Ser396 and Ser422) through the activation of GSK3β, JNK, and ERK 1/2 kinases [4].

Recent epidemiological studies showed that two-thirds of AD patients are women [5], and this fact cannot be attributed only to their higher life expectancy. In this connection, the loss of estradiol might be one of the factors leading to declining cognitive function in women [6].

Of note, we found that oxidative stress, together with important oxidative stress-related risk factors related to AD, such as hypoxia, hyperglycemia, and hypercholesterolemia, are potential causes of the increased BACE1 activity [7]. In AD, estrogen neuroprotective activity is exerted at multiple levels. Preclinical data showed that, in addition to their action against neuroinflammation and oxidative stress, estrogens are able to influence both the main players of neurodegeneration, Aβ, and tau [8].

In this paper, we pursue the hypothesis that biological sex influences the effect of $A\beta_{42}$ monomers on pathological tau conformational change. Our data revealed that $A\beta_{42}$ monomers produce the pathological conformational changes and hyperphosphorylation of tau protein in male or ovariectomized female mice but not in control female. The treatment of ovariectomized females with estradiol replacement protects against the pathological conformation of tau. The hypothesized protective mechanism is mediated both by their antioxidant activity and by their ability to modulate the expression of miRNA 218 linked to tau phosphorylation.

3'), mouse tau gene (forward
CCCACCTGTAAC-3', reverse
GTATGTCCACCC-3'), and di
(forward 5'-CAGGCTTTGAA
reverse 5'-TGAACTTGTGGC
3'). Mice were maintained on
129/SvJae/C57BL/6 backgroun
were kept on a 12 h light/dark
and water available *ad libitum*.
procedures on live animals were
the supervision of a licensed vete
to: 1) European Communities
(November 24, 1986; 86/609/
Ministry of Health and Universit
tutional guidelines on animal w
on Care and Protection of living a
experimental or other scientific
rization No. 17/2010-B, June 30
hoc Ethical Committee of the U
(http://www.unito.it/unitoWAR/pa
ricerca1/Ricerca_comitato1).

Two groups of 2-month-old m
mice were treated for 3 h ($n = 60$
O_2/N_2O anesthesia, hTau mice
injected with Aβ peptides or s
used for injection were: anterop
lateral, 1.2 mm relative to Breg
tral, 1.7 mm from the dural surfac
validated by injecting one mouse
(1 μl).

Ovariectomy

Two groups of 2-month-old fe
went bilateral ovariectomy (OV
sham-operated groups. The bilater
thetized female mice were expose
incision in the dorsal skin and n

centration) with sterile double distilled water, centrifuged at 10000 g for 10 min to remove possibly aggregates and then intraventricularly injected. The quality of Aβ preparations was controlled using atomic force microscopy (AFM). AFM was carried out on a Multimode AFM with a Nanoscope V system operating in Tapping Mode using standard antimony(n)-doped Si probes (T: 3.5–4.5 mm, L: 115–135 mm, W: 30–40 mm, f0:313–370 kHz, k: 20–80 N/m) (Bruker). The scan rate was tuned proportionally to the area scanned and was kept in the 0.5–1.2 Hz range. The sample was then diluted to 5 μM with PBS, and 50 μl of solution was spotted onto a freshly cleaved muscovite mica disk and incubated for 5 min. The disk was then washed with ddH_2O and dried under a gentle nitrogen stream. Samples were analyzed with the Scanning Probe Image Processor (SPIP Version 5.1.6 released April 13, 2011) data analysis package (Nanoscience Instruments, Phoenix, AZ, USA). SPIP software was used to analyze the distribution of the molecular assemblies of the different populations in terms of height and diameter, as previously described [10]. Our controls were hTau mice ICV injected with saline. The experiments were done four weeks after ovariectomy. After two weeks from ovariectomy surgery, one group of female mice was subjected to a daily subcutaneous injection of 17β-estradiol (E_2) for three weeks (1 μg/Kg) [11]. Animals were allowed to recover for at least three weeks before experiments were performed and subsequently were subjected to intracerebroventricular injection of $A\beta_{42}$ monomers (200 nM) or saline and sacrificed after 3 h.

Antibodies and immunoblot analysis

Immunoblot analysis was performed using the following antibodies: MC1 (kind gift from Dr.

150 mM NaCl, 1 mM EGTA, 1 m
PMSF, phosphatase and proteas
then centrifuged at 10,000 g fo
to isolate soluble proteins. Supe
solution) were collected and inc
gent sarkosyl (5% final concentra
4°C. The sarkosyl mixtures wer
in Beckman SW 55 Ti rotor a
1 h at 4°C. Pellets were resus
sample buffer to obtain sarkosyl-
Lysates (20 μg) were run on 3–
dient PAGE gel (Invitrogen) an
to nitrocellulose membrane. Bl
(5% no fat milk) and incubated
with primary antibodies. Peroxida
ondary antibodies were incuba
temperature (RT) and develope
Forte Western substrate (WB
pore). Densitometric values we
β-actin.

Total antioxidant capacity

To evaluate the antioxidant cap
tissues, we performed the total an
(TAC) dosage kit (ab65329, Abc
was performed on total extracts acc
ufacturing protocol.

Quantitative determination of 17β

To quantify E2 levels, blood mi
obtain plasma. To perform the me
commercially available ELISA kit
alog # ADI-901-174) according to
protocol.

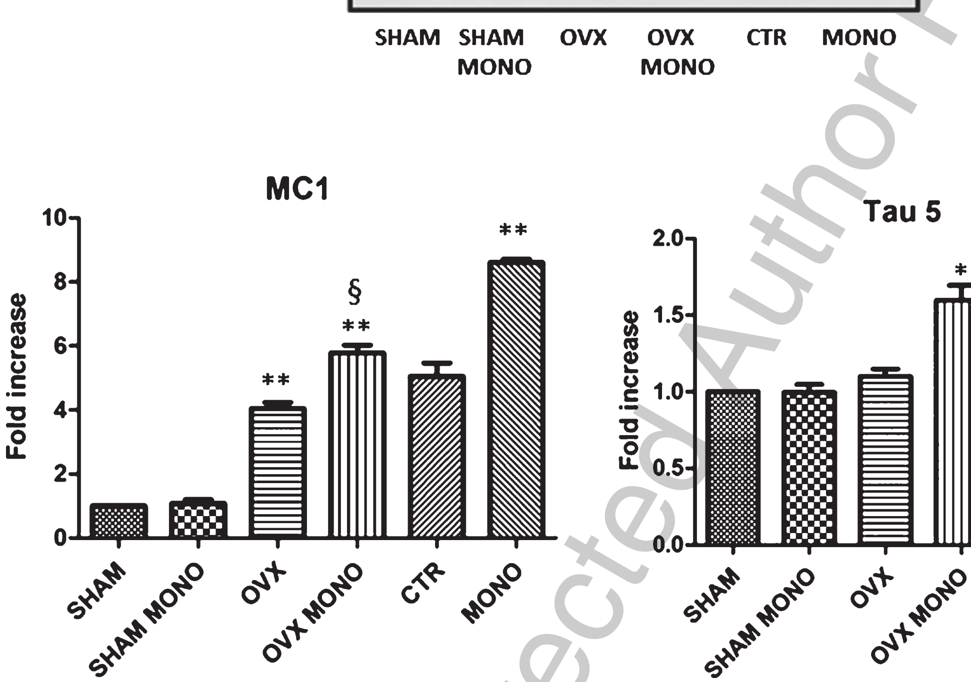

Fig. 1. Intracerebroventricular treatment with Aβ_{42} causes a change in tau conformation only in male or ovarie
Representative western blot of brain samples from control (saline) and treated Aβ_{42} peptides for ICV male and fe
conformational tau antibody (MC1) and a total tau antibody (Tau 5) for detection. Some female mice were subjected
served as loading control. Densitometric quantification shows an increase of the total protein level of both MC1
ovariectomized female mice injected or not with Aβ_{42}. The data are mean ± standard error of the mean (SEM); *p <
control; §$p < 0.05$ versus OVX by one-way ANOVA followed by Bonferroni post test $n = 5$.

MicroRNA isolation and quantitative real time PCR

MicroRNA was isolated from brains of female mice using the MagMAXmirVana kit and according to manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Subsequently, cDNA synthesis was performed using the TaqMan Mi-croRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and a RT-primer pool con-

taining microRNA-specific stem
miR218 (Mature miRNA Sequen
GAUCUAACCAUGU) and for sn
Sequence: GTGCTCGCTTCG
ACTAAAATTGGAACGATACA
CATGGCCCCTGCGCAAGGAT
TCGTGAAGCGTTCCATATTTT

Each qPCR contained 1.3 μL
1 μL 20X TaqMan MicroRNA
2X TaqMan Universal PCR Ma

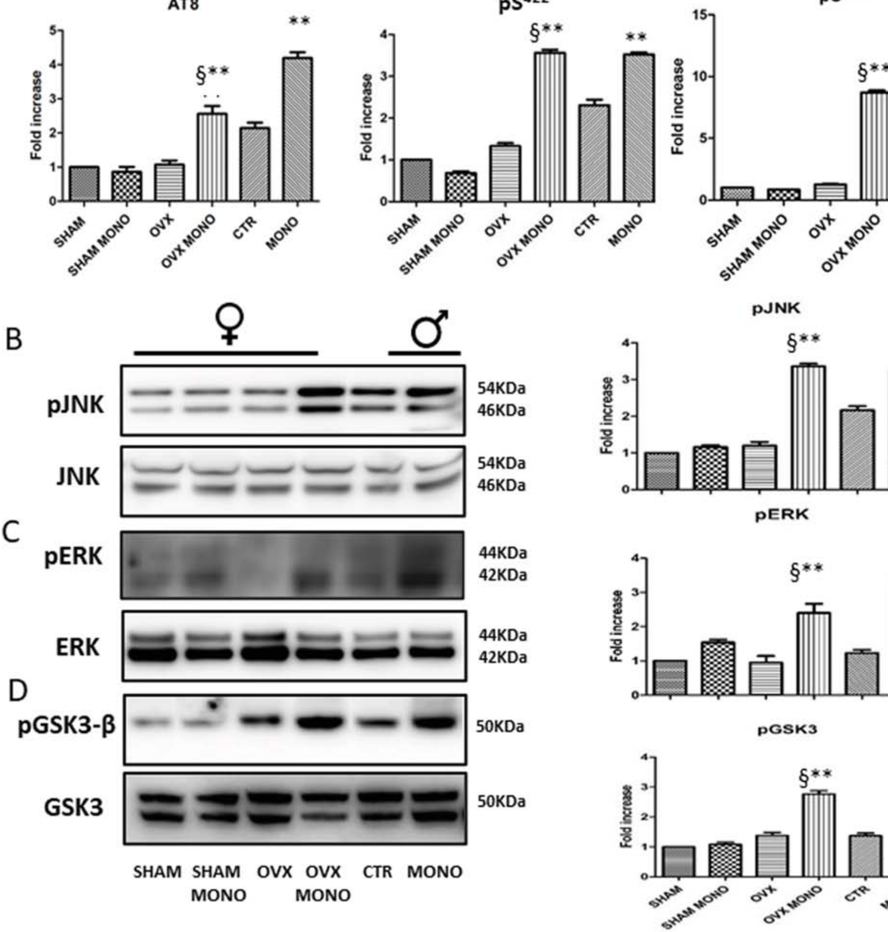

Fig. 2. The conformational change mediated by $A\beta_{42}$ is induced by protein hyperphosphorylation. A) Representati
extracts from control (saline) and treated $A\beta_{42}$ peptides for ICV male and female hTau mice using antibodies spec
pathological tau phosphorylation sites such as AT8, pS422, and pS396. Some female mice were subjected to ovarie
loading control. Densitometric analysis shows a significant increase of total protein levels of AT8, pS422, and pS39
ovariectomized female mice. B–D) Representative western blot of brain extracts from control (saline) and treated
male and female hTau mice using p JNK (B), pERK1/2 (C), and pGSK3β (D). Some female mice were subjected
served as loading control. Densitometric analysis shows a significant increase of total protein levels of all the three ki
and ovariectomized female mice. The data are mean ± standard error of the mean (SEM); $^{*}p < 0.05$; $^{**}p < 0.01$ v
versus OVX by one-way ANOVA followed by Bonferroni post test $n = 5$.

All values were presented as mean ± standard error of the mean (SEM). Means were compared by one or two-way analysis of variance (ANOVA) with Bonferroni as a *post-hoc* test. Values of $^{*}p < 0.05$ were considered significant, $^{**}p < 0.01$ very significant, and $^{***}p < 0.001$ extremely significant.

RESULTS

Intracerebroventricular treatment with Aβ$_{42}$ causes a change in tau conformation only in male or ovariectomized female mice

Figure 1 shows that, as previously demonstrated, the intracerebroventricular injection of 200 nM Aβ$_{42}$ in male hTau mice is able to determine a pathological conformational change of the tau protein. The same treatment is able to determine this effect only in female mice after ovariectomy. The same result was obtained by evaluating the total tau protein levels. As can be seen, treatment with Aβ$_{42}$ significantly increases total tau levels in male (1.5-fold increase) and ovariectomized female mice (1.5-fold increase) with respect to control female mice injected or not with Aβ1$_{42}$.

The conformational change mediated by Aβ$_{42}$ is induced by protein hyperphosphorylation

To understand if the conformational change of tau was due to hyperphosphorylation, we measured the phosphorylation of some specific sites related to the pathology by western blotting. Phosphorylation levels were studied through the use of the AT8 antibody (which recognizes the Ser 202/Thr 205 epitopes), the antibody S396 and S422. These are all phosphorylation sites closely associated with disease progression.

(+3-fold increase). A similar resu
measuring the nuclear levels of p
which increase in the same grou
times compared to the controls (F

Estradiol hormone therapy protec
Aβ$_{42}$-mediated tau conformationa

To confirm that the presenc
involved in the different effect ex
ment with Aβ$_{42}$ on the pathologi
change of tau, groups of female mi
or not were subcutaneously trea
(E_2) (1 μg/kg) and fed with a soy
weeks. We first tested the validity
measuring the levels of estradiol i
groups. As can be seen, oophorec
decreases circulating estradiol lev
E2 treatment determines an incr
becomes significantly higher with
females (+100%) (Fig. 3). Figure
treatment with estradiol complete
pathological conformational chan
of total tau mediated by Aβ$_{42}$
females. Then, to further confirm
of estradiol, we studied the ins
sarkosyl detergent technique, and
a tau band at approximately 75 kD
revealed with Tau 46 antibody
Aβ$_{42}$ in ovariectomized female, w
with estradiol blocks the aggrega
(Fig. 4B).

Estradiol therapy protects female
against Aβ$_{42}$-mediated tau hyperp

Figure 5 shows that enrichm
is also followed by complete pr

Fig. 3. Estradiol hormone therapy significantly increases E_2 levels. Groups of female mice, ovariectomized or not, were subcutaneously treated with E_2 (1 μg/kg) and fed with a soy-free diet for three weeks. To test the validity of the treatment, we measured E2 levels in serum of our experimental groups. We observed that the ovariectomy induces a significant decrease in hormone levels, whereas the treatment protects the decrease of E2 levels, that significantly increased with respect to controls. The data are mean ± standard error of the mean (SEM); $*p < 0.05$; $**p < 0.01$ versus control by one-way ANOVA followed by Bonferroni post test $n = 6$.

mediated phosphorylation, after oophorectomy, of pathology-related sites (Fig. 5A). As expected, the kinases involved in the phosphorylation of the above sites do not appear induced; thus, as observed in Fig. 5B, the levels of nuclear pJNK, pERK, and pGSK3β are absolutely comparable to the levels of the control females.

Estrogen hormone therapy protects male hTau mice against $A\beta_{42}$-mediated tau conformational change and hyperphosphorylation

To further confirm the protective role of estradiol on the pathological conformational change of tau and its hyperphosphorylation, we also treated hTau male mice with estradiol, following the same protocol as the females. As can be seen in Fig. 6, the treatment with estradiol is able to completely protect both the conformational change of tau as revealed by the significant increase of the band revealed with MC1 antibody as well as its hyperphosphorylation revealed by using AT8 antibody that recognizes Ser202/Thr205 phospho-epitopes (Fig. 6).

and the simultaneous intracerebr
tion of $A\beta_{42}$ induces a further d
parameter (–70%). Treatment wit
the drop-in antioxidant capacity b
to control values (Fig. 6A).

Finally, we measured levels
recent discoveries demonstrate th
tors are able to modulate the expres
involved in tau phosphorylation [
has been found that increase of m
level of target protein tyrosine p
consequent enhancement of tau p

We observed that levels of miR2
higher in ovariectomized female m
with $A\beta_{42}$, whereas the E2 treatm
a total protection of the miRNA
It is interesting to note that the
male hTau mice treated with Aβ a
obtained in females after oophorec

DISCUSSION

In our work we pursued the hyp
influences the effect of $A\beta_{42}$ mon
cal tau conformational change. Ou
$A\beta_{42}$ produced pathological conf
and hyperphosphorylation of ta
or ovariectomized female mice
female. The risk of developing
progression and severity are know
different in men and women [14
the drop-in estrogen and the path
by data indicating that early me
the risk of developing dementia
cognitive decline is greater in w
at the same stage of disease an
correlated with estrogen levels [1
the therapeutic potential of estro

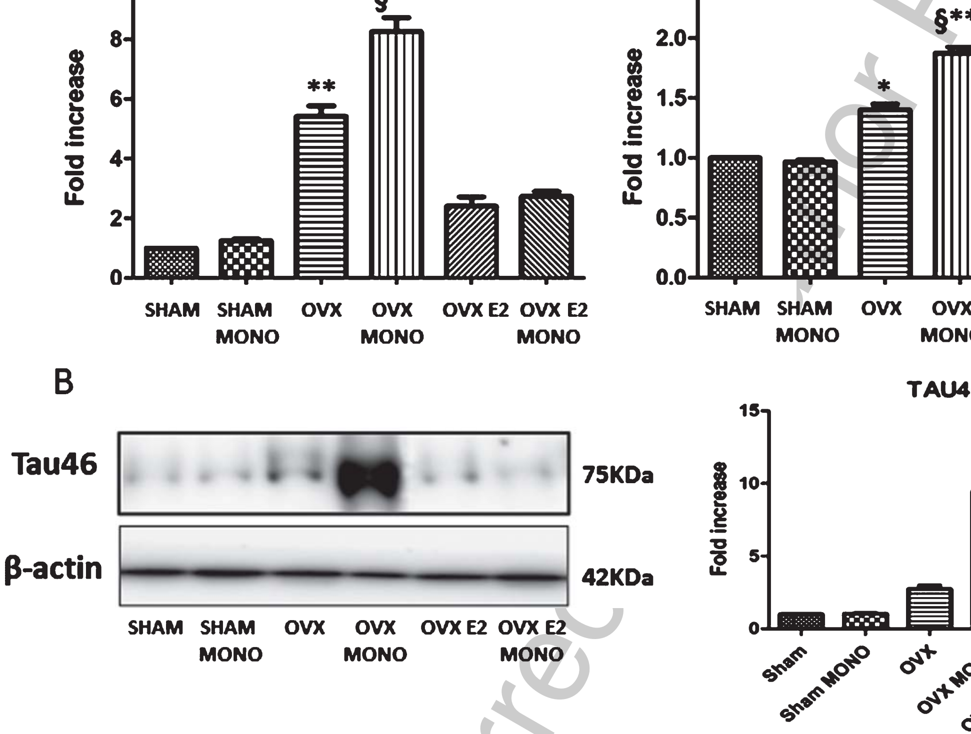

Fig. 4. Estradiol hormone therapy protects against $A\beta_{42}$-mediated tau conformational change. A) Representativ
samples from control (saline) and treated $A\beta_{42}$ peptides for ICV female hTau mice using a conformational tau anti
tau antibody (Tau 5) for detection. Some female mice were subjected to ovariectomy and or to E_2 (1 μg/kg) and
weeks. β-actin served as loading control. Densitometric quantification shows an increase of the total protein level
in ovariectomized female mice injected or not with $A\beta_{42}$; the treatment with estradiol completely protects the con
the increase of tau protein. B) Representative western blot of insoluble tau fraction by sarkosyl detergent techniqu
(saline) and treated $A\beta_{42}$ peptides for ICV female hTau using Tau 46 antibody for detection. β-actin served as loading
of $A\beta_{42}$ in ovariectomized females, we showed a band at approximately 75 kDa molecular weight revealed with Ta
estradiol blocks the aggregation of tau protein. The data are mean ± standard error of the mean (SEM); $^{*}p < 0.05$; **
$^{\S}p < 0.05$ versus OVX by one-way ANOVA followed by Bonferroni post test $n = 5$.

active in the last years and recently it has taken new life, thus finding new therapeutic approaches for AD is one of the most important challenges of modern medicine. Numerous experimental evidences have

shown that estrogens have protec
induction of neuroinflammation
ation [16–18]. The encouraging
vitro clashed with clinical trials c

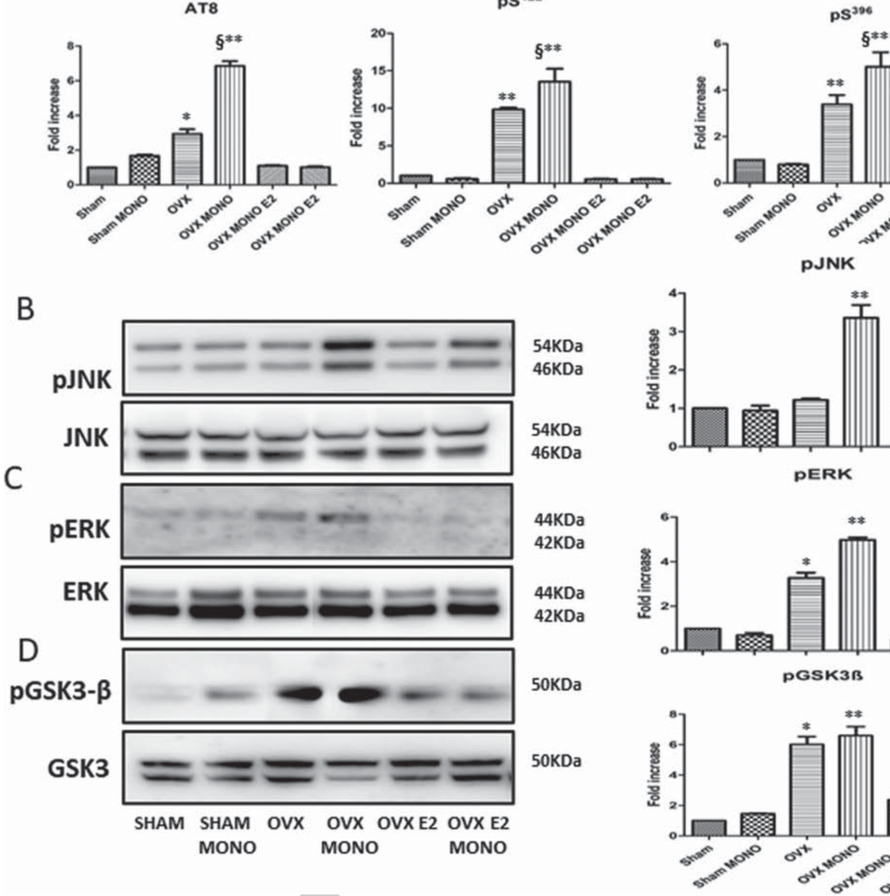

Fig. 5. Estradiol hormone therapy protects against Aβ_{42}-mediated tau hyperphosphorylation. A) Representative weste
from control (saline) and treated Aβ_{42} peptides for ICV female hTau mice using antibodies specific for the detect
phosphorylation sites such as AT8, pS422, and pS396. Some female mice were subjected to ovariectomy and or to E_2
diet for three weeks. β-actin served as loading control. Densitometric analysis shows an increase of total protein lev
pS396 in ovariectomized female mice injected or not with Aβ_{42}; the treatment with estradiol completely protects th
of tau protein. B–D) Representative western blot of brain extracts from control (saline) and treated Aβ_{42} peptides fo
using p JNK (B), pERK1/2 (C), and pGSK3β (D). Some female mice were subjected to ovariectomy and or to E_2 (1
for three weeks. β-actin served as loading control. Densitometric analysis shows an increase of total protein levels
in ovariectomized female mice injected or not with Aβ_{42}; the treatment with estradiol completely protects the ac
The data are mean ± standard error of the mean (SEM); $^*p < 0.05$; $^{**}p < 0.01$ versus control; $^{\S}p < 0.05$ versus OV
followed by Bonferroni post test $n = 5$.

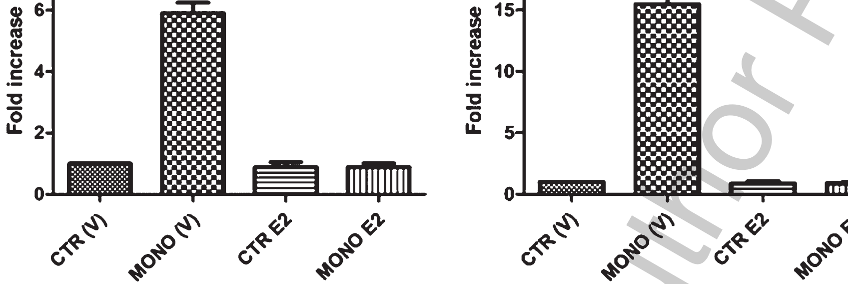

Fig. 6. Estradiol hormone therapy protects male mice against Aβ_{42}-mediated tau conformational change as well as
Representative western blot of brain samples from control (saline) and treated Aβ_{42} peptides for ICV male hTau mice
tau (MC1) and a specific antibody for tau pathological phosphorylation (AT8) for detection. Some male mice were su
and soy-free diet for three weeks. β-actin served as loading control. Densitometric quantification shows an increase
of both MC1 and AT8 in mal mice injected or not with Aβ_{42}; the treatment with estradiol completely protects the con
the increase of tau phosphorylation. The data are mean ± standard error of the mean (SEM); $^{**}p < 0.01$ versus contr
followed by Bonferroni post test $n = 5$.

clinical trials had shown that the use of replacement therapy significantly increased the risk of dementia and cognitive decline [19, 20]. These studies suggested that patients aged 65 and over, already in menopause for a long time, did not represent an adequate experimental group, because replacement therapy is indicated for women who have just gone through menopause, and suggested the presence of a therapeutic window useful for this type of therapeutic approach [21]. More recently, emerging evidence suggests that protein tau could be a potential target for estrogens. It has been demonstrated that 17β estradiol promotes tau dephosphorylation *in vitro* in rat cortical neurons and SH-SY5Y neuronal cells [22]. Other authors confirmed these results showing that E2 prevent the phosphorylation of tau in an estrogen receptor-mediated and dose-dependent manner [23]. *In vivo* studies have shown that estrogenic treatment increases GSK3β phosphorylation in Ser 9/21, a site that inactivates the kinase activity, protecting the phosphorylation of pathological sites related to

disease progression [24]. Moreo
suggest that estrogens exert thei
through their alpha-type recepto
with insulin-like growth factor 1
by incorporating itself into a mac
plex that includes phosphoinosit
and protein kinase A (AKT). The
signal pathways leads to an inh
and therefore to a reduction in
tion [25]. Our results confirm t
of estrogens on the pathological
hyperphosphorylation of tau med
broventricular injection with Aβ
mice. From our results, it cann
if estradiol acts as an antioxida
therefore, in a mode independe
or modulates at the receptor leve
pathways. We showed that it in
that, under Aβ treatment, hyper
these results suggest a cellular
estradiol.

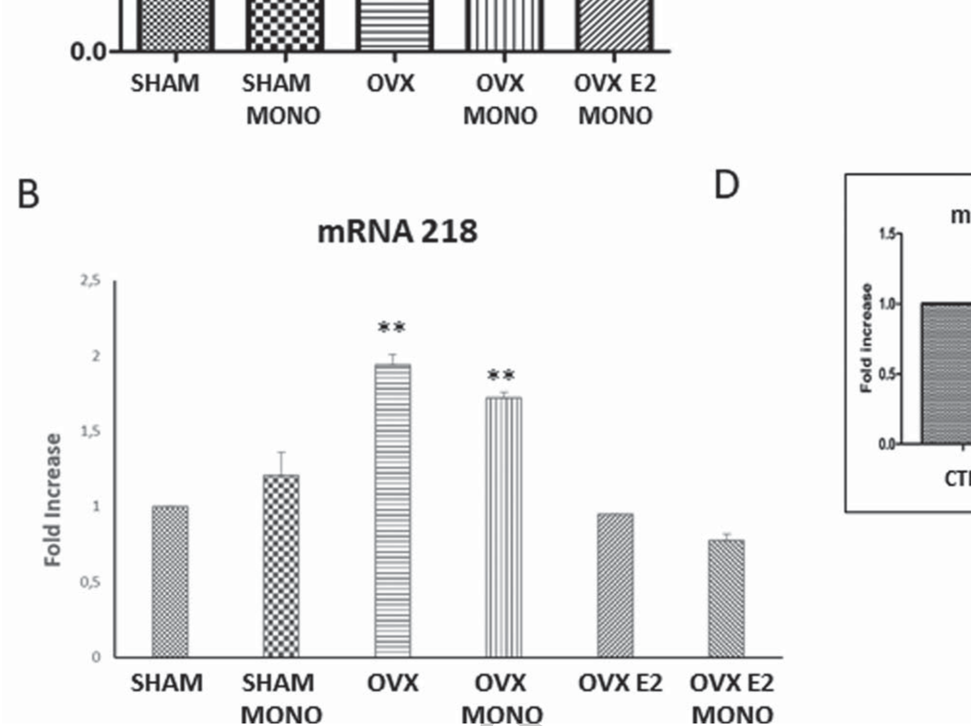

Fig. 7. Estradiol hormone therapy protects against oxidative stress and downregulate miRNA 218. A) Evaluation
capacity in female mice subjected to ovariectomy and or to E_2 (1 μg/kg) and soy-free diet for three weeks. We
significantly decreases the antioxidant capacity respect to control females, whereas the treatment with estradiol protects
capacity. B) miRNA 21 levels capacity in female mice subjected to ovariectomy and or to E_2 (1 μg/kg) and soy-fr
We show that ovariectomy significantly increases the levels of miRNA 218 with respect to controls female, the t
completely protects the increase. C) Evaluation of the total antioxidant capacity in male mice treated or not with Aβ
decreases the antioxidant capacity respect to control males. D) miRNA 21 levels capacity in male mice treated or not w
$A\beta_{42}$ significantly decreases miRNA level respect to control males. $*p<0.05$; $**p<0.01$ versus control; $^{\S}p<0.05$ v
ANOVA followed by Bonferroni post test $n=5$.

A lot of protective mechanisms relating to estrogens have been described in the literature [26]. Most recent studies have revealed that estrogens exert an antioxidant action not only by direct chemical neutralization of reactants, but also by modulating the expression of antioxidant enzyme of biological reducing agents [27] tal models, we found that the dec levels was followed by a decrease idant activity and that this event with important oxidative stress-related risk factors related to AD such as hypoxia, hyperglycemia, and hypercholesterolemia, are potential causes of the increased BACE1, the crucial enzyme for Aβ production, activity [7].

Recently emerging evidence suggests that estrogens are involved in regulation of microRNAs in many pathological conditions [28]. Rao et al. showed that estradiol regulates particular target miRNAs in a specific tissue and age manner in ovariectomized rats [29]. Furthermore, the deprivation of estrogens caused the progressive loss of regulation of the miRNA, leading to a lack of regulation even after the reintroduction of the estrogens [30]. In our work we focused our attention on the miRNA 218, because it is implicated in the phosphorylation of tau upon estrogen receptor (ER) α and β activation. There are two known ERs, usually referred to as ERα and ERβ, and both are widely distributed in the brain [31]. In the brain of patients with AD, both ERα and ERβ are defective. Mitochondrial ERβ is reduced in the frontal cortex of female patients with AD [32], and the alternative splicing of ERα mRNA is decreased in the AD brain especially in female patients [33]. Moreover, in the hippocampus of AD patients the ERα-expressing neurons are reduced [34], whereas ERβ immunoreactivity is increased [35]. These findings indicate a potential role of these two receptors in the pathogenesis of AD. Then it has been reported that the neuroprotection against Aβ toxicity by estrogens requires the expression of both receptors and the activation of mitogen-activated protein kinase pathway [36]. Specifically, Xiong et al. demonstrated opposite effects of these two receptors on tau phosphorylation. ERα overexpression increased miRNA 218 expression and the hyperphosphorylation of tau, whereas ERβ decreased miRNA 218 expression and tau phosphorylation [13]. Interestingly, a number of miRNA

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Authors' disclosures availabl
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